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(54) Title: MEMBRANE FUSION EVENTS AND MEANS FOR ALTERING SAME

(57) Abstract

The present invention relates to a method of altering (reducing or enhancing) membrane fusion events in a wide variety of contexts, including infection of cells by organisms (such as pathogens including viruses and particularly influenza virus), cell-cell interactions or fusions (such as cell signalling and sperm-ovum union) and intercellular membrane fusion events. It further relates to agents useful in the method of altering membrane fusion events and to a method of identifying or designing agents which alter membrane fusion events.

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MEMBRANE FUSION EVENTS AND MEANS FOR ALTERING SAME

Description

Related Application

This application is a Continuation-in-Part of U.S. Serial No. 08/005,223, filed January 15, 1993, by Chavela M. Carr and Peter S. Kim, the contents of which are hereby incorporated by reference.

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Background of the Invention

The selectively-permeable lipid bilayer of biological membranes preserves the topologically distinct environments required by cells and by eukaryotic organelles and other subcellular compartments. Many cellular processes require mixing of the contents of distinct membrane-bound environments, which, in turn, requires that membrane fusion occurs. The fusion of distinct membranes to form a single lipid bilayer is essential for many events, both intracellular and intercellular. Membrane fusion between cells is required, for example, for union of sperm and egg, and for cell signaling, such as neurotransmission. In addition, membrane fusion is associated with many disease states, such as the

entry of all enveloped viruses into host cells, and the virally-induced cell-cell fusion caused by the human immunodeficiency virus (HIV), which results in cell death. Membrane fusion, however, is very slow in the absence of the specific proteins, and requires machinery specialized for the specific membrane fusion event. The components of that machinery and their interactions are presently not well understood.

Summary of the Invention

The present invention relates to a method of altering (reducing or enhancing) membrane fusion events in a wide variety of contexts, including: infection of cells by organisms such as pathogens, including viruses and particularly influenza virus; cell-cell interactions or fusions, such as cell signalling and sperm-ovum union; and intercellular membrane fusion events. It further relates to agents or drugs (referred to collectively as agents) useful in the method of altering membrane fusion events, and to a method of identifying or designing agents or drugs which alter membrane fusion events. In the present method, a membrane fusion event is altered (reduced, partially or totally, or enhanced) by interfering with a conformational change in a molecule, herein referred to as a fusion protein, that results in or is associated with membrane fusion. This is carried out by exposing the fusion protein to (combining or contacting it with) an agent or drug which alters the conformational change in the protein, or by exposing it to a condition (such as a change in temperature) which alters the conformational change.

In one embodiment, the present invention relates to a method of reducing the membrane fusion of an enveloped virus that is essential for infection of a host cell, propagation of the virus within a host cell, or both, and, thus, of reducing infection of host cells by the virus. In a specific embodiment, the present invention relates to a method of reducing the infectivity of influenza virus by reducing or preventing the conformational change that Applicants have identified and characterized as occurring in

influenza hemagglutinin (in the influenza fusion protein). In this embodiment, the influenza virus is contacted with an agent or drug which alters the conformational change (e.g., prevents the conformational change, alters the extent, timing and/or location of its occurrence, or permits conformational change to occur but not to result in the conformation Applicants have determined the virus assumes for membrane fusion). In another specific embodiment of the current invention, the extent to which membrane fusion occurs between the human immunodeficiency virus (HIV) and host cells is reduced, thereby reducing the infectivity of the virus, by reducing or preventing the conformational change necessary for membrane fusion. The present invention also relates to agents or drugs useful in preventing the conformational change.

In another embodiment of the present method, membrane fusion mediated by a molecule (e.g., a protein, such as a cell surface protein) is altered (enhanced or reduced) by altering the conformational change undergone by the fusion protein. "Enhancement", in this context, means increasing the extent, duration or level of the conformational change, relative to its extent, duration or level in the absence of the present method. "Reduction" indicates decreasing the extent, duration or level of the conformational change, relative to its extent, duration or level in the absence of the present method. In a specific embodiment, the extent to which membrane fusion occurs between sperm and ovum is enhanced by contacting the sperm and/or ovum with an agent or drug which increases the conformational change of a sperm fusion protein, an ovum fusion protein or both. For example, the sperm cell surface fusion protein PH-30 can be assessed for conformational change associated with membrane fusion. An agent or drug which enhances the PH-30 conformation change can be administered, resulting in enhanced joining of sperm and egg (enhanced fertilization). Conversely, an agent which reduces sperm-ovum membrane fusion can be used to reduce, partially or totally, fertilization. Such agents or drugs can be administered

vaginally in biologically acceptable carriers.

Brief Description of the Drawings

Figure 1 is a schematic diagram of receptor-mediated endocytosis cell entry by a virus.

Figure 2 is the amino acid sequence of BHA2. The amino acid sequence of BHA2 is shown under the heptad repeat (abcdefg). The amino-acid sequence, which is proposed to form an extended coiled coil at how pH, is underscored. The fusion peptide and LOOP-36 peptides are indicated by a bar above the sequences.

Figure 3 is a schematic representation of the structure of BHA in the dormant state.

Figure 4 is a helical wheel representation of 28 residues with high coiled-coil propensity. The view is down the helical axis starting at the N-terminus. The peptide begins and ends at the f position. The alignment of hydrophobic amino acids at the a and d positions and hydrophilic amino acids at other positions reveals the strikingly amphipathic nature of the sequence.

Figure 5 is a series of graphs representing folding of LOOP-36. Open circles = neutral pH; filled circles = low pH. 5(A): Circular dichroism (CD) spectroscopy at 0°C indicates a characteristic α-helical spectrum (>90% helix content based on the value of $[\theta]_{222}$) for LOOP-36 at pH 4.8. In contrast, the CD spectrum for LOOP-36 at pH 7.0 indicates a random-coil conformation. 5(B): The CD signal at 222nm for LOOP-36 shows a cooperative, thermal-unfolding transition at pH 4.8. In contrast, no transition is seen for LOOP-36 at pH 7.0. 5(C): The molecular weight of LOOP-36 as determined by sedimentation equilibrium experiments. The slopes of the lines shown are proportional to molecular weight. The average molecular weight at pH 4.7 was 13.6 kD (expected for trimer = 13.4 kD), while the measured molecular weight at pH 7.2 was 5.2 kD (expected for monomer = 4.5 kD).

Figure 6 is a series of graphs representing folding of LOOP-52. Open squares = neutral pH; filled squares = low

pH. 6(A): Circular dichroism (CD) spectroscopy at 0°C indicates a characteristic α-helical spectrum (>95% helical based on the value of $[\theta]_{222}$; however, values obtained in the 0.01 cell are only good to within ~10%) for LOOP-52 at pH 4.8 and pH 7.0. 6(B): The CD signal at 222nm for LOOP-52 at ~500 μM shows a cooperative, thermal-unfolding transition which is reversible at both pH 4.8 and pH 7.0. The basellines of the two samples are offset due to a slight difference in peptide concentration. 6(C): The molecular weight of LOOP-52 as determined by sedimentation equilibrium experiments. The slopes of the lines shown are proportional to molecular weight. The average molecular weight at pH 4.7 was 19.26 kD, and the measured molecular weight at pH 7.2 was 19.98 kD (expected for trimer = 18.4 kD).

Figure 7 is a graphic representation showing pH-dependence of LOOP-36 folded structure.

Figure 8 is a graphic representation of the results of molecular weight determination of LOOP-36.

Figure 9 is a schematic representation of a spring-loaded trigger model for the conformational change of hemagglutinin; for clarity, the HA1 subunits are omitted. Figure 10 demonstrates the helical-hairpin structure in Hemagglutinin (HA): 10(A) is a representation of the helical-hairpin structure in the trans-membrane subunit (HA2) of HA, with the C-terminal residues omitted for clarity; 10(B) is a representation of the interaction of the HA1 domain with the HA2 helical hairpin in an HA monomer.

Figure 11 is a schematic diagram of the structural units in HA2 at pH7, with the sequences of the LOOP-36 (residues 54-89) and LOOP-52 (residues 38-89) underscored.

Figure 12 is a graphic representation of the LOOP-36 folding transition in a physiologically relevant pH range. The pH-transition for LOOP-36 folding is superimposed on published results for the pH transitions of HA conformational change and membrane fusion activity. The CD signal at 0°C for a sample of LOOP-36 was monitored at 222nm as a function of pH (filled circles). Two transitions are observed. The transition at ~pH 3 probably is not physio-

logically relevant. The transition at ~pH 5.7 agrees well with other pH transitions reported for HA. A sharp transition is seen between pH 7 and pH 5 for viral-cellular membrane fusion (open squares) (Wharton et al., Virology 149:27-35 (1986), exposure of a fusion peptide epitope to a monoclonal antibody (plus signs) (White et al., J. Cell Biol. 105:2887-2896 (1987), protease sensitivity of BHA (open circles) (Ruigrok et al., J. Gen. Virol. 69:2785-2795 (1988), liposome binding by BHA (X) (Doms et alal., J. Biol. Chem. 260:2973-2981 (1985), dissociation of HA1 subunits and exposure of an epitope to a monoclonal antibody (open triangles) (White et al., J. Cell Biol. 105:2887-2896 (1987), and cell fusion by BHA (open diamonds) (Doms et al., J. Biol. Chem. 260:2973-2981 (1985)).

Detailed Description of the Invention

The present invention is based on Applicants' study of membrane fusion events, which has resulted in a better understanding of how these events occur, as well as in a model for conformational change which occurs in a component of the fusion machinery and makes the fusion event possible. Applicants' work has far-reaching applicability, in that membrane fusion is essential for a wide variety of biological events, including intracellular events, such as protein trafficking, and intercellular events, such as cell-cell fusion, cell signaling and viral infection.

As described herein, Applicants have identified a conformational change which occurs in a viral envelope polypeptide necessary for fusion of membranes; the change renders the polypeptide, and particularly a certain portion of the polypeptide, available to mediate fusion of the host cell endosome membrane and the viral membrane (the envelope). As a result of the conformational change, a peptide previously thought to participate in membrane fusion but, according to previous models, unable to mediate the fusion event because of structural constraints, is extended toward the membrane with which fusion is to occur and, thus, is available to participate in or mediate the fusion event.

In particular, Applicants have determined that hemagglutinin (HA), the influenza virus envelope glycoprotein necessary and sufficient for membrane fusion at low pH, or at neutral pH and high temperature, is a coiled coil structure; and that this structure plays an important role in the conformational change which hemagglutinin undergoes at lower pH, particularly the lower pH (approximately 5.0) which occurs in the later endosome, or at neutral pH and higher temperature. As used herein, the term "fusogenic state" describes the conformation at the lower pH or at the neutral pH and higher temperature, that is necessary for membrane fusion. The term "dormant state" or "metastable state" describes the conformation at neutral pH and/or non-elevated temperature present when membrane fusion does not normally occur. Applicants' work, described herein, supports the model that the conformational change undergone by hemagglutinin to reach the fusogenic state acts much like a spring-loaded trigger which projects the fusion peptide away from the viral envelope and toward the endosomal membrane. The extension of the three-stranded coiled coil relocates the fusion peptide toward the endosomal membrane, relieving the steric constraints on the fusion peptide which appeared to confine it near the viral envelope in previous models of the fusion event. As a result, the fusion peptide is available to mediate membrane fusion.

The following is a description of Applicants' investigation of the influenza hemagglutinin protein, their surprising finding that hemagglutinin is a coiled-coil structure, and their model of the conformational change undergone by hemagglutinin at the fusogenic state, which results in the extension of hemagglutinin fusion peptide toward the endosomal membrane with which fusion occurs. The following is also a description of methods of altering membrane fusion events, such as those which occur between an enveloped virus and a host cell membrane, and, thus, of altering (reducing) infection of cells by the virus, based on knowledge of the conformational change, as well as of agents or drugs useful as antiviral agents or drugs.

Further, the following is a description of how other membrane fusion events, such as cell-cell fusion caused by the human immunodeficiency virus, or fusion of sperm and ovum, can be reduced or enhanced by altering (reducing, enhancing or changing the timing and/or location of) the conformational change which occurs in fusion proteins necessary for such fusion events. Even further, it is a description of an assay system and method useful for identifying agents or drugs which alter (reduce or enhance) membrane fusion events in which a conformational change in a necessary fusion protein. Because of the importance of membrane fusion in so many, diverse cellular events and cellular interactions, Applicants' findings have widespread applicability and offer new approaches to altering fusion events.

Membrane Fusion as Exemplified by Influenza Virus Infection

Influenza Virus In General

A well-characterized membrane fusion event occurs in the infection of animal cells by influenza virus. The influenza virion binds to proteins displayed on the surface of the host cell and is internalized by receptor-mediated endocytosis as a virion encapsulated by a cellular endosome. (See Wiley, D.C. et al. "Viral Membranes" In Virology, ed. Fields, Raven, New York (1990) and Figure 1). Initially, the internal pH of the endosome is neutral; as the endosome matures, the internal pH is lowered to approximately pH 5. The change in pH activates a membrane fusion reaction between the viral membrane (the envelope) and the membrane of the endosome; this reaction is mediated by a viral glycoprotein. The same membrane fusion can also be induced at neutral pH, at temperatures above 60°C (Ruigrok et al., Virology 155:484-497 (1986)). In addition, mutants which induce fusion at higher pH have ben analyzed, and a striking correlation is seen between the increase in pH of fusion and a decrease in temperature required to induce fusion at neutral pH (Ruigrok et al., Virology 155:484-497 (1986)).

Membrane fusion results in release of the viral genome into the cytoplasm, where replication and propagation of the virus occur.

The resulting virions are released from the host cell and infect additional host cells, in which they undergo the same processing. For influenza, the viral component responsible for viral-endosomal membrane fusion at the fusogenic state is the trimeric, viral-envelope glycoprotein, hemagglutinin (HA), which also functions in viral attachment at neutral pH by binding to sialic acid, a component of host cell membrane proteins. The conformation of HA at neutral pH, in which HA can bind to sialic acid, is herein referred to the "dormant state" or "metastable state". Membrane fusion results from destabilization of the metastable conformation of HA by either low pH, or elevated temperature at neutral pH. The HA protein is necessary and sufficient for the fusion of membranes in vitro and in vivo.

In the metastable state, HA folds as a symmetric trimer. Each monomer consists of a disulfide-bonded pair of polypeptides, designated HA1 and HA2, which are proteolytically cleaved from a precursor, HA0, which folds and assembles as a homotrimeric membrane protein (Ward, C. W. et al., Aust. J. Biol. Sci. 33:449-455 (1980)). The amino-terminal cleavage product, HA1, is extracellular and associates with the other two HA1 subunits of the trimer to form the sialic acid binding sites. The three HA1 subunits assemble on top of a fibrous stem, which is formed primarily by the interaction of three HA2 polypeptides (see Figure 10). Each HA2 subunit spans the membrane once and contains the agent responsible for membrane fusion at its amino terminus. The length of the trimer is approximately 135 Å from the junction with the membrane to the top of the HA1 subunits (Wilson, I. et al., Nature 289:366-373 (1981)).

The HA2 polypeptide folds into a helical hairpin-like structure (see figure 10): a short α-helix is connected to a long α-helix by an extended loop. The long α-helix interacts with the long α-helix of two other HA2 polypeptides to form an interwound rope of three helices, called a

three-stranded coiled-coil. The three shorter α-helices are displayed outside the coiled-coil. HA2 is the transmembrane subunit: each HA2 polypeptide spans the envelope membrane once. The amino terminus of HA2, derived from the cleavage site of HA0, begins with a highly conserved, hydrophobic sequence of approximately 25 amino acids, which, in the dormant state, is buried near the viral membrane in the interface of the three-stranded coiled coil. This sequence has been implicated in membrane fusion; thus, it is called the "fusion peptide". The fusion peptide becomes exposed after the conformational change in HA, in the fusogenic state. The fusion peptide becomes inserted into the endosomal membrane (Stegmann, et al., EMBO J. 9:4231-4241 (1990)) and are known to be necessary for membrane fusion at low pH (Daniels et al., Cell 40:431-439 (1985); (Gething et al., J. Cell Biol. 102:11-23 (1986)). Removal of the fusion peptide eliminates HA-mediated membrane fusion in vivo and in vitro (Ruigrok, R.W.H. et al., J. Gen. Virol. 69:2785-2795 (1988).

The extracellular domain of HA can be cleaved from the viral membrane by bromelin to release the exoplasmic domain from the viral membrane. The resulting soluble domain, termed BHA, possesses the same sialic acid-binding and lipid-binding functions as intact hemagglutinin. In the metastable state, the structure of BHA is a symmetric trimer of three globular subunits atop a fibrous stem. The BHA1 polypeptide folds into a globular β-sheet domain that forms each BHA1 subunit. The BHA2 polypeptide folds into a fibrous, helical-hairpin stem that associates with the two other BHA2 subunits as a three-stranded coiled coil. The helical-hairpin structure of BHA2 starts at the amino terminal residues 1-34, followed by the exposed α-helix, residues 35-53, the loop, residues 54-89, and the long, internal α-helix of the coiled coil, residues 90-127. The fusion peptide (residues 1-25, included in the amino terminal residues 1-34) is buried in the trimer interface of the coiled coil. The x-ray crystal structure of BHA has been determined at neutral pH, to 3 Å resolution, with and

without sialic acid bound. The hemagglutinin-sialic acid interaction has been well described and is separate from the function of HA in membrane fusion.

Activation of fusion of the viral membrane with the endosome membrane is known to require a conformational change in hemagglutinin in order to release the fusion peptide from the interior of the trimer. There is considerable evidence for a large conformational change, which not only exposes the fusion peptide, but also results in the dissociation of the HA1 subunits. Doms, R.W. et al., J. Biol. Chem. 260:2973-2981 (1985); Ruigrok, R.W.H. et al., J. Gen. Virol. 69:2785-2795 (1988); White, J. et al., J. Cell Biol. 105:2887-2896 (1987); Darnell, J. et al., Molecular Cell Biology, Scientific American Books, pp. 573-575, W.H. Freeman and Co., New York (1990). The conformational change is induced by mildly acidic conditions (e.g., at ~pH 5, the pH of the mature endosome) and results in a trimeric structure that is remarkably thermostable (Doms and Helenius, J., Virol 60:833-839 (1986); Ruigrok et al., Virology 155:484-497 (1986)). At neutral pH, a similar conformational change, leading to a stable fusion-active state of HA, can be induced at temperatures above 60°C (Ruigrok et al., Virology 155:484-497 (1986)).

Until now, the structure of the conformation of hemagglutinin in the fusogenic state has not been known. Thus, a basic question has remained unanswered: how does the fusion peptide mediate membrane fusion if it is constrained by the structure of the protein to be 35 Å from the viral envelope and at least 100 Å from the endosomal membrane? Applicants have studied the structure of the influenza virus hemagglutinin and the conformational change which occurs, and have determined that hemagglutinin undergoes a conformational change which acts much like a spring-loaded trigger to extend the fusion peptide toward the endosomal membrane, bringing it in sufficient proximity to the membrane to mediate the fusion event necessary for further viral processing and infection of additional host cells.

The following is a simple model for the conformational

change in influenza hemagglutinin that occurs as the molecule undergoes conformational change to the fusogenic state. Results of work described support the discovery that in the fusogenic state, the hemagglutinin fusion peptide is projected toward the endosomal membrane atop a long, three-stranded coiled coil. As a result, the fusion peptide is available to mediate membrane fusion. The model arose out of Applicants' interest in the two-stranded coiled coil structure motif found in the "leucine zipper" domain of transcription factors and in the fibrous proteins myosin, tropomyosin and keratin. Analysis of the influenza hemagglutinin showed, surprisingly, that it is a coiled coil structure and that the coiled coil structure plays an important role in the previously uncharacterized conformational change undergone by hemagglutinin at lower pH (pH of approximately 5.0, which is that of the late endosome), or at neutral pH and elevated temperature.

Hemagglutinin is a Coiled Coil Structure

The coiled-coil motif forms a rope structure of interwound α-helices with a left-handed superhelical twist. Crick, F.H.C., Acta. Cryst. 6:689-697 (1953). The coiled-coil amino-acid sequence can be identified by a characteristic degenerate consensus sequence which is repeated in tandem. In general, a heptad of hydrophobic and hydrophilic amino acids, denoted as positions a through g (see Figure 4), has apolar amino acids at positions a and d and polar amino acids at the other positions. Hydrophilic residues tend to occur at positions a and d of the heptad repeat, and form the interface between helices. This repeat of apolar amino acids is known a the "4-3 hydrophobic repeat," which is a hallmark of coiled coils. The frequency of occurrence of each amino acid at each position in the heptad repeat has been tabulated for both two and three-stranded coiled coils. Parry, D.A.D., Biosci. Rep. 2:1017 (1982); Conway, J. F. et al., Int. J. Biol. M. 13:14-16 (1991).

An algorithm based on the frequency of occurrence of

amino acids in two-stranded coiled-coils was used to identify coiled-coils in known protein sequences. (Lupas, A. et al., Science, 252:1162-1164 (1991)). This analysis revealed a continuous, 88-residue sequence in HA2 that has an unusually high score, based on the coiled-coil algorithm. Surprisingly, the highest-scoring sequence was shown not to be within the known three-stranded, coiled-coil structure, but to begin at the N-terminal of the short or-helix, and to encompass the entire, extended loop region, which connects the short, external α-helix and the long α-helix of the structurally known coiled-coil. This sequence corresponds to a region of low antigenic variation among hemagglutinins of different strains of influenza virus (Wiley, D.C. et al., Ann. Rev. Biochem. 56:365-394 (1987)).

Because of the coiled-coil, it is hypothesized that HA2 folds into a long, three-standard coiled-coil in the fusogenic state (Figure 9); in response to a drop in pH (or, at neutral pH, an increase in temperature), the coiled-coil is extended to include the LOOP region and the short, external-helix. Thus the 80 Å coiled-coil that exists in the dormant state forms a 135 Å coiled-coil in the fusogenic state. Thus, it appears to provide a region of the virus which can be a common target for agents designed or selected to interfere with membrane fusion, as an approach to reducing infection.

A Model for the Conformation Change of Hemagglutinin

These considerations led to the following hypothesis for the conformational change of hemagglutinin: in the fusogenic state, the loop region becomes helical and associates as a three-stranded coiled-coil. This in turn brings the short α-helices together as a three-stranded coiled-coil (see Figure 3). Thus, the 80 Å coiled-coil of 52 amino acids in the metastable state is extended to form a 140 Å coiled coil of 92 amino acids, in the fusogenic state. This conformational change projects the fusion peptide away from the viral envelope and toward the endosomal membrane. As a result, the fusion peptide is extended 100 Å toward the

endosomal membrane, relieving the steric constraints on the fusion peptide which, in previous models, confine the fusion peptide to be near the viral envelope. This results in the positioning of the fusion peptide in sufficient proximity to the membrane for it to be able to mediate the fusion event.

To further investigate this model and assess its applicability, two polypeptides (LOOP-36 and LOOP-52) were constructed, and analyzed under conditions causing the conformational change from the metastable (dormant) state to the fusogenic state. First, a 36-residue peptide (LOOP-36, Figures 2 and 4) which corresponds to the 28 amino acids of the loop region, plus 8 residues of the long α-helix of HA2 in the dormant structure, was synthesized and analyzed as to its conformation under fusion conditions, involving low pH (approximately 5.0). A prediction of this particular model is that the peptide will form a three-stranded coiled coil in the fusogenic state. This prediction is stringent for the following reasons: with the exception of coiled coils, in aqueous solutions, peptides of naturally occurring sequences do not fold into stable structures of a conformation different from that found in the native protein; examples of peptides that fold into "non-native" structures are rare; and there are very few precedents for drastic conformational changes between two distinct folded structures.

In the fusogenic state (i.e., under conditions of lower pH, or of neutral pH and raised temperature), LOOP-36 is folded and helical; at the metastable state (i.e., under conditions of neutral pH and usual temperature), it is in an unfolded, random-coil conformation. Secondary structure was monitored by circular dichroism (CD) spectroscopy. At 0°C, the CD spectrum of LOOP-36 has the shape of a highly helical structure at pH 4.8 and of a random coil at pH 7.0 (Figure 5A). Reversible thermal unfolding of LOOP-36 revealed a cooperative transition at pH 4.8 and no unfolding transition at pH 7.0 (Figure 5B).

Two cooperative transitions are revealed by monitoring the helicity of LOOP-36 as a function of pH. A very sharp transition is seen from a structure of >90% helicity at

100mm at pH 4.8 to a complete loss of helicity at pH 6 and higher. No stable structure exists at neutral pH. The Predominance of acidic residues at particular positions in the LOOP-36 sequence (see Figure 4) is reminiscent of the arrangement seen in the FOS leucine zipper homodimer, which displays a similar pH-dependence of stability (O'Shea et al., CELL 68:699-708 (1992)). Protonation of acidic side chains at low pH alleviates electrostatic repulsion that destabilizes the folded on conformation at neutral pH. A second transition is seen between pH 2 and pH 4: as the pH is decreased below pH 4 there is a loss of helicity to ~50% at pH 2 (Figure 7). Thus transition is probably irrelevant for membrane fusion.

LOOP-36 is a trimer in the fusogenic state and a monomer in the metastable state (Fig. 5C). Molecular mass was determined by equilibrium centrifugation. At pH 4.7 the average mass is 2% higher than expected for a trimer, and at pH 7.2 the mass is 16% higher than expected for a monomeric peptide (Figure 8 and the Table).

TABLE

HMG-1 Equilibrium Centrifugation

pH	Molecular Weight
4.7	Expected: 13,350 Da (for trimer) Average: 13,633 Da
7.2	Expected: 4,450 Da (for monomer) Average: 5,172 Da

The composition of amino acids in the structure may assist the pH-related conformational change. The numerous polar and charged amino-acids in the HA2 sequence may favor the hairpin structure and prevent the loop from forming a coiled coil at neutral pH. These and/or other amino acids may have opposite effects at low pH, i.e., to destabilize the hairpin and stabilize the coiled coil. Additionally, there are likely to be interactions which stabilize the

fusion peptide buried in the trimer interface at pH 7 which are destabilizing at low pH, to facilitate the release of the fusion peptide at the pH of fusion.

There is a striking similarity between the pH transition observed for LOOP-36 helicity, the known pH dependencies for the conformational changes and for the onset of membrane fusion activity in HA (Fig. 12). Exposure of the fusion peptide, dissociation of the HA1 subunits, liposome binding, cell-cell fusion, and viral fusion have been monitored as a function of pH (Doms et al., J. Biol. Chem. 260:2973-2981 (1985); White et al. J. Cell. Biol. 105:2887-2896 (1987); Ruigrok et al., J. Gen. Virol. 69:2785-2795 (1988)); all of these studies indicate a sharp transition between pH 7 and 5. While correlation may suggest some role for the loop region in the mechanism for the conformational change at low pH and at temperatures below 60°C, that role is likely to be minor, even fortuitous, in light of studies involving LOOP-52.

The transition from the metastable state to the fusogenic state was also examined at neutral pH with elevated temperatures. Because induction of the fusogenic state occurs at high temperatures as well as at low pH, it was suspected that, at neutral pH, coiled-coil structure in the loop region may be stabilized in the context of the longer coiled coil. to test this notion, a 52-residue peptide, LOOP-52, which includes LOOP-36 and the residues of the short, external α-helix (Fig. 11), was studied. In the crystal structure of the dormant state of HA, only the C-terminal 8 residues are in a coiled-coil conformation.

LOOP-52 forms a fully helical (~100%) structure at pH 7.0 and pH 4.8 (Fig. 6A). At both neutral pH and pH 4.7, LOOP-52 is a trimer, as determined by equilibrium sedimentation (Fig. 6C). The trimeric, helical structure in LOOP-52 is very stable: at pH 7.0, LOOP-52 unfolds with a transition midpoint of ~60°C, and at pH 4.8, the transition midpoint is ~75°C (Fig. 6B).

Because LOOP-52 forms a stable, α-helical trimer, even at pH 7, coiled-coil formation by the loop region does not

require low pH in the content of a longer coiled coil. This conclusion is consistent with the observation that HA-mediated fusion can occur at neutral pH (Ruigrok et al., Virology 155:484-497 (1986)). Furthermore, monoclonal antibodies (mAb) specific to either hemagglutinin conformation at the neutral pH or the hemagglutinin conformation low pH show HA1 dissociation and exposure of the fusion peptide with a similar pH dependence as that seen for the folding of LOOP-36.

These results demonstrate that the loop region can be stabilized in the context of the longer coiled-coil at neutral pH; provide strong support for the proposal that the coiled-coil in the fusogenic state includes the external α-helix in addition to the loop region; and indicate that the longer coiled coil is very stable, even at neutral pH. Proteolytic analysis further supports the hypothesis. In the metastable state, intact BHA is resistant to proteolysis, but in the fusogenic state, the pattern of proteolysis reveals that the HA1 subunits move away from the HA2 stem, and the fusion peptide is exposed to proteolysis. Most strikingly, in the fusogenic state, the final proteolytic product of BHA2 begins with the first amino acid of the proposed, extended coiled coil and extends to the C-terminus of BHA2. The residues of the short α-helix, Loop region, and the long α-helix remain intact. These results would come as a surprise if the loop were extended in the low-pH conformation, because it should have been cleaved by proteases, as the HA1 subunits are no longer associated in this conformation to protect the loop from exposure to proteolysis. Instead, the loop region is protected from proteolysis, as would be expected if it were part of a folded structure, such as a coiled-coil.

Also consistent with the current model for the conformation of the fusogenic state, HA is trimeric at pH 5 (Doms and Helenius, J. Virol. 60:833-839 (1986)). In addition, electron microscopic studies have suggested formation of an extended fibrous structure on the surface of influenza viral membranes, following treatment at low pH or elevated

temperatures at neutral pH (Ruigrok et al., Virology 155:484-497 (1986); Ruigrok et al., J. Gen. Virol. 69:2785-2795 (1988)).

The current model thus suggests a strong propensity for the loop region of HA2 to form a coiled coil at the fusogenic state. However, it is clear from mutational analysis that the amino acids in the vicinity of the buried fusion peptide and a buried region of HA1 are important for the conformational change that exposes the fusion peptide and the HA1 region. Mutants of HA which fuse membranes at elevated pH were selected by the chemical elevation of the internal pH of the endosome. The majority of the mutants in the HA2 polypeptide are either hydrophobic amino-acid substitutions in the fusion peptide sequence or polar substitutions in the regions of the protein surrounding the buried fusion peptide and buried HA1 region. Thus, they are thought to reveal interactions which secure the fusion peptide and the HA1 region in the protein interior at neutral pH.

There is another interpretation of the mutagenesis results. Most of the mutations in HA2 fall within the 88 amino-acid sequence which are predicted herein to form a coiled coil at low pH. The charged and polar nature of the wild-type and substituted amino acids may point to those which are important for stabilizing the hairpin structure over the coiled coil at neutral pH and/or the coiled coil over the hairpin at low pH. For example, two HA2 mutants replace charged amino acids in the LOOP-36 sequence: glutamate 81 to glycine, and lysine 58 to leucine; these charged positions may be important for the pH-induced conformational change of HA2 from a hairpin structure to a three-stranded coiled coil. The contribution of specific amino acids to the coiled coil structure at low pH can be dissected by amino-acid substitutions in LOOP-36. Testing of the role of amino acids in stabilizing the hairpin, requires making the 88 residue peptide. If the peptide folds into a helical hairpin trimer at neutral pH, substitutions can be made to identify the amino acids

involved in stabilizing this structure at neutral pH. These considerations suggest that the release of the proposed spring-loaded trigger involves a complex set of interactions which can be dissected by site-directed mutagenesis.

Comparison of the Current Model to Other Models

Current models for the activation of HA address published evidence for the exposure of the fusion peptide, dissociation of the HA1 headgroups and little change in secondary structure. The fusion peptide is envisioned to swing out from the stem, but remain close to the viral envelope, as the HA1 subunits fall away from the stem of the trimer. Most models also invoke the formation of a multi-HA pore complex to account for electron micrographs of virions at low pH that have rings of HA on the surface of the viral envelope, and electrophysiological studies which suggest the formation of pores during membrane fusion. (White, J.M., Science 258:917-924 (1992)). At low pH, the HA trimer is envisioned to associate as a pore complex of HA molecules in which each HA trimer is bent sideways in order to facilitate endosomal-membrane contact by the fusion peptide. Alternatively, the fusion peptide is thought to aid in mixing with the endosomal membrane by coating the center of the pore complex, allowing lipids to cross between the viral envelope and the endosomal membrane.

In contrast, Applicants' work is the basis for the prediction that the fusion peptide is projected away from the viral envelope and toward the membrane of the endosome. In Figure 9, the BHA2 trimer is depicted as three spring-loaded helical hairpins, bent at the loop and fastened by the N-terminal "belt" with the hydrophobic fusion peptide as the "catch" which holds the helical hairpin in the bent conformation. The BHA1 dissociate is omitted from Figure 9 for clarity. As the molecule moves from the metastable state to the fusogenic state, the extended, three-stranded coiled coil is the preferred conformation; the fusion peptide "catch" is released from the protein interior and extended 100 Å toward the target membrane atop a long, three-stranded

coiled coil. Figure 9 is a schematic representation of Applicants' model, referred to as a spring-loaded trigger model, for the conformational change of hemagglutinin. The extension of the three-stranded coiled coil relocates the fusion peptide 100 Å toward the endosomal membrane, relieving the steric constraints on the fusion peptide which confine it to be near the viral envelope in other models. The results of work with LOOP-36 and LOOP-52 provide evidence for the conformational change predicted by the model. It is unlikely that the coiled coil forms in the fusogenic state as the result of an artifact, since it folds into a stable structure which is distinct from that seen at the metastable state, and stable, non-native structures are rare, especially for small peptides, as discussed above.

The Mechanism for the Conformational Change of the Current Model

The current model for the fusogenic structure of HA suggests a "spring-loaded" mechanism for the conformational change that mediates membrane fusion. The existing three-stranded coiled coil in the dormant state is extended to include the loop region and the short α-helix (Fig. 9). This conformational change relocates the N-terminal fusion peptide 100 Å from its position near the viral membrane to its position in the target membrane. As a result, HA2 becomes an integral membrane protein in both the viral and the endosomal membranes. In this manner, the loop is activated to form a coiled-coil and the fusion peptide is released from the protein interior; one might envision the following sequence of events: first, the loop is made competent to fold into a coiled coil (spring-loading the trigger, in effect); next, the fusion peptide, acting as a "catch," is released; dissociation of the HA1 subunits then occurs, resulting in the capacity for liposome binding; and finally, membrane fusion occurs. Hydrophobic peptides are known to associate spontaneously with membranes; thus, once released, the fusion peptides of the trimer penetrate the endosomal membrane, forming a trimer that is an integral

membrane protein in both the lipid bilayer of the viral envelope and the endosomal membrane. Such trimers may associate to form a pore complex which is more efficient in securing the two bilayers together and that encompasses a region devoid of other membrane proteins which might prevent lipid mixing.

Others have postulated mechanisms for lipid mixing and membrane rupturing that require a hydrophobic lining of the pore complex (see White review: Ann Rev. Physiol. 52:675-697 (1990)). Achieving such hydrophobicity would require a subsequent conformational change or dissociation of the three-stranded coiled coil, as the amino acids which comprise the a and d positions are hydrophobic, and most of those at the other positions are charged and polar. One possibility involves the dissociation of the coiled coil into individual, amphipathic α-helices and the interaction of the hydrophobic face of the helices with the hydrophobic tails of the endosomal lipids. Thus, the trimers would disperse laterally as the membranes fuse.

A protein conformational change of the magnitude proposed here, while rare, is not unprecedented. A striking example, demonstrated by x-ray crystallography, involves the serpin family of protease inhibitors. Upon cleavage of the reactive center peptide bond, a segment of these proteins can undergo a dramatic conformational change in secondary and tertiary structure, resulting in the relocation of a short region by 70 Å (Wright et al., J. Mol. Biol. 213:513-528 (1990); Stein et al., Nature 347:99-102 (1990); Mottonen et al., Nature 355:270-273 (1992)).

Even though LOOP-52 is very stable at the neutral pH, formation of the long coiled coil is prevented in the in vivo folding and assembly of the HAO precursor. It is proposed, however, that the dormant state produced alter proteolytic processing of HAO is metastable (i.e., though it is stably folded, it has the potential to form an even more stable state).

Biophysical studies have shown that the thermal transition corresponding to the induction of fusion at

neutral pH is irreversible; this irreversible transition is followed by a second, reversible transition at even higher temperature (Ruigrok et al., Virology 155:484-497 (1986)). These results support two major assertions of our hypothesis for the mechanism of the conformational change. First, the irreversible nature of the first transition (which corresponds to induction of fusion) is consistent with the metastable nature of the dormant state. Second, the reversible transition at higher temperature is likely to be the unfolding transition of the long coiled coil, consistent with the reversible unfolding transition seen in LOOP-52 at neutral pH.

There appear to be two major constraints that inhibit formation of the more stable fusogenic state: (i) inter-subunit protein-protein interactions and (ii) burial of the fusion peptide in the hydrophobic core of the timer. In the structure of the dormant state, extensive interactions between the loop region of HA2 and its corresponding HA1 subunit are apparent (Fig. 10B). Thus, the HA1 subunit may stabilize the helical hairpin structure of the dormant state by acting as an inhibitor or "clamp" that binds to the loop region, preventing the conformational change. In addition, multiple HA1-HA1 interactions are likely to contribute to the stability of the dormant state. Indeed, introducing disulfide bonds between HA1 subunits inhibits the conformational change in HA and prevents membrane fusion (Godley et al., Cell 68:635-645 (1992)). Furthermore, many mutations in HA, selected to mediate fusion under less acidic conditions, appear to destabilize HA1 interactions in the dormant state (e.g., Ruigrok et al., Virology 155:484-497 (1986)).

At low temperature (0°C), however, there is evidence for HA-mediated membrane fusion in the absence of HA1 dissociation (Stegmann et al., EMBO J. 9:4231-4241 (1990)) This would suggest that membrane fusion is promoted by a conformation of HA that is intermediate between the crystal structure and the extended coiled-coil conformation that we propose. Alternatively, completed dissociation of HA1 may

not be required for extension of the coiled coil, or fusion may be promoted in these experiments by a small population of molecules with dissociated HA1 subunits.

The metastable conformation of the helical hairpin may also be constrained by the fusion peptide, which makes significant hydrophobic interactions in the core of the dormant structure. The buried fusion peptide resembles a hydrophobic "hook," which holds the helices together in a helical hairpin conformation (Fig. 1A). Many of the HA mutants selected for their ability to fuse under less acidic conditions contain amino acid substitutions near the region of the fusion peptide. The x-ray crystal structure of the fusion mutant D112G, for example, reveals that 4 hydrogen bonds are lost (per monomer) between the aspartate side chain and residues of the fusion peptide. The pH of fusion for the mutant is elevated by 0.4 pH units, presumable because loss of the hydrogen bonds destabilizes the metastable conformation of the dormant state.

Mutations that affect fusion by altering the stability of the fusogenic state are expected to be rare, since the long coiled coil is very stable even at neutral pH. The metastable hypothesis predicts that, because fusion is triggered by destabilization of the dormant state, mutatns that affect the stability of the dormant state will be more common. Indeed, there is a good correlation between the decrease in stability and the increase in the pH of fusion by the HA mutants (Ruigrok et al., Virology 155:484-497 (1986)).

It is notable that conformational change similar to that described here has been proposed recently for a transcription factor. A transition from monomer to trimer in yeast heat shock factor (HSF) is seen with an increase in temperature (Rabindran et al., Science 259:230-234 (1993)). Although the structures have not been determined for either monomer or trimer of HSF, it is also possible that "spring-loaded" mechanisms exist in proteins that are not involved in membrane fusion.

Other Viral and Nonviral Membrane Glycoproteins

Influenza hemagglutinin is the best characterized membrane fusion protein; however, other viral and non-viral membrane glycoproteins share similar features. Trimeric and tetrameric membrane-fusion glycoproteins have been identified, and some of these conform to a set of criteria which makes them candidates for further study in light of the model. A few of these protein sequences are proteolytically processed from a precursor and contain a fusion peptide-like sequence at the N-terminus created after proteolysis. The model of the current invention suggests that the 4-3 hydrophobe repeats following these fusion peptides may correspond to a coiled-coil structure that is found only in the fusogenic conformation of the polypeptide.

Viruses which fuse in the endosome may share the same pH-induced mechanism for the conformational change as that of an hemagglutinin-like fusion protein. The proteins which mediate pH-neutral fusion events (such as the fusion of the sperm with the ovum, or HIV with T cells) may undergo the same type of conformational change in response to an external stimulus, such as in response to ligand binding or loss of protein-protein interactions. The interactions made between the extracellular subunits and the transmembrane domains in the case of proteins which mediate pH-neutral fusion events may also undergo the same type of conformational change. Based upon the current model, it is reasonable to expect that a similar conformational change occurs in the case of both types or groups of fusion proteins (i.e., those, such as influenza hemagglutinin, which participate in a pH-induced event; or a different protein which is induced by a pH-neutral event, or even a protein in which a change in pH is not the triggering or conformational change-inducing event).

For example, the binding of HIV to CD4 is known to induce conformational changes in the envelope glycoprotein that result in release of the extracellular subunit (gp120) from the transmembrane subunit (gp41), exposure of

the fusion peptide, and fusion of the viral membrane with the cell (reviewed in Vaishnav and Wong-Staal, Ann. Rev. Biochem 60:577-630 (1991)). A peptide from gp41, corresponding to a sequence adjacent to the N-terminal fusion peptide, has recently been shown to form a coiled coil (Wild et al., PNAS USA 89:10537-10541 (1992)). Moreover, expression of gp41 in the absence of gp120 results in syncytium formation (Perez et al., J. Virol. 66:4134-4143 (1992)), demonstrating that the interaction between gp120 and gp41 inhibits fusogenic activity.

Methods of Altering Membrane Fusion Events and Agents Useful in Altering Same

Knowledge of the conformational change undergone by a protein which mediates membrane fusion events can be used to identify or design of agents or drugs which alter (reduce, enhance and/or change the timing or location of) the conformational change and, as a result, alter (reduce, partially or totally, or enhance) the fusion event(s) which the fusion protein mediates. In particular, knowledge of the conformational change undergone by hemagglutinin and metastable conformation of the protein can be used to identify and design agents or drugs useful in reducing infection by the influenza virus. In addition, assessment of the fusion event and conformation of other fusion proteins (e.g., those of viral pathogens, non-viral pathogens, other organisms, animal cells, including mammalian and particularly human, cells) before and during membrane fusion can be carried out in a manner similar to that described herein for an enveloped virus (i.e., influenza virus). The resulting understanding of conformational changes can be used to identify and/or develop agents or drugs useful to reduce or enhance membrane fusion and, as a result, reduce or enhance the outcome (e.g., host cell infection, fertilization of an ovum, cell signaling, cell-cell fusion and protein trafficking) which would occur in the absence of the agent or drug.

For example, therapeutic agents can be designed to

prevent the activation of hemagglutinin for membrane fusion and subsequent viral reproduction. An agent, drug, or compound (e.g., an antibody, peptide, small organic molecule) designed to bind irreversibly and stabilize the metastable state of the LOOP-36 peptide would prevent the conformational change and subsequent release of the viral genome into the host cell for viral replication and propagation. Preventing the conformational change in this manner can be used to reduce or prevent infectivity of viruses other than influenza as well. An agent, drug or compound designed to bind irreversibly and stabilize the metastable state of the fusion protein of the virus would prevent the conformational change and subsequent release of the viral genome into the host cell.

Alternatively, agents which cause premature release of the spring-loaded trigger and, thus, prevent the virus from binding to cellular receptors and entering the host cell via receptor-mediated endocytosis, can be identified or designed. As used herein, "premature release" or "premature induction of the conformational change" refers to the occurrence of the conformational change at a time prior to that at which the change would occur in the absence of the agent. For example, an agent which stabilizes the fusogenic state, or destabilizes the dormant state of HA, might release the fusion peptide prematurely to abolish the sialic-acid binding function of HA, and decrease or eliminate infectivity of the virus. For influenza hemagglutinin, prevention of the conformational change can be brought about simply by maintaining pH at neutral pH or at least above the pH at which the conformational change occurs (e.g., above approximately pH 5.0), and maintaining the temperature below 60°C. Premature induction of the conformational change or induction at a location other than that at which the conformation change normally occurs (in the late endosome) can be brought about, for example, by lowering the pH of the cells/tissues in which the influenza virus resides, prior to its being present in the late endosome, or by raising the temperature of the

cells/tissues. This can be effected, for example, by exposing the cells to heat, or administering agents which maintain or lower pH; dilute acetic acid or other acid can be administered by known methods, such as nasally. In either case, membrane fusion will not be possible and further infection will be reduced, totally or partially. Premature induction of the conformational change can also be used in this manner to prevent infectivity of enveloped viruses other than influenza. An agent triggering premature induction would inactivate the virus permanently, and would not have to be delivered into the cell in order to be active.

In the case of hemagglutinin, LOOP-36 can be used in an assay to determine the effectiveness of a known or a newly designed agent in altering the conformational change of hemagglutinin. LOOP-36 is a trimer, which is folded and helical, in the fusogenic state and is a monomer, which is an unfolded, random-coil conformation, in the metastable state. Change in its conformation (from the metastable form to the fusogenic form) in the presence of an agent or a drug being assessed can be used as an indication of the ability of the agent or drug to prevent induction of the conformational change associated with membrane fusion (and, thus, to reduce influenza virus infection and propagation). If conformational change does not occur at conditions under which the molecule would move into the fusogenic state in the presence of the agent or drug, it is indicative of the agent or drug's ability to reduce the conformational change and, as a result, to reduce or prevent infection. For example, LOOP-36 can be combined with an agent or drug under conditions of neutral pH and the pH then lowered (e.g., to approximately that of the late endosome), or under conditions of neutral pH and then the temperature raised (e.g. to approximately 60°C, at which the molecule usually enters the fusogenic state). If, in the presence of the agent or drug and at low pH, LOOP-36 retains its metastable (dormant) conformation (or assumes a conformation different from either the metastable conformation or the fusogenic

conformation, with the result that the fusion peptide is not released or available to mediate membrane fusion), it is indicative of the ability of the agent or drug to reduce or prevent the change and, thus, reduce or prevent infection. Alternatively, a similar assay can be used to identify agents which cause premature induction of the conformational change from the metastable (dormant) state to the fusogenic state. If the conformational change occurs prior to the time when it would occur in the absence of the agent or drug, it is indicative of the ability of the agent or drug to cause premature induction of the conformational change, and as a result, to reduce or prevent infection. For example, if, in the presence of the agent or drug, LOOP-36 undergoes the conformational change prematurely, it is indicative of the ability of the drug to reduce or prevent infection.

An assay similar to this assay can also be used to identify agents which can either prevent induction of the conformational change, or cause premature induction, and thus reduce or prevent infection of any enveloped virus. For example, the fusion protein or fusion molecule of the virus in question can be combined with the agent or drug to be tested under conditions under which the conformational change would normally occur. If, in the presence of the agent or drug, the fusion protein either remains in the metastable (dormant) state or prematurely enters the fusogenic state, it is indicative of the ability of the agent or drug to alter the conformational change, and thus, reduce or prevent infection. For example, HIV gp120 can be assessed for conformational changes which occur in virus-mediated cell-cell fusion events. It can be combined with agents or drugs to be assessed and conformational change (or lack/reduction thereof) assessed in a similar manner as for LOOP-36, using the methods described herein. In the case of sperm-ovum fusion, similar experiments can be conducted to isolate agents or drugs which enhance or reduce fusion. Accordingly, a fusion peptide which mediates sperm-egg fusion (e.g., PH-30, a sperm surface protein) can be

included in an assay to assess the ability of agents or drugs to reduce or enhance its conformational change and, thus, to reduce or enhance sperm-egg fusion (fertilization). Change in conformation can be assessed using methods described herein and other known methods.

Exemplification

A. Materials and Methods

Coiled Coil Prediction

An algorithm (Parry, 1982) was used to predict coiled-coil propensities based on the statistical preference of each amino acid for each position in the heptad repeat. Sequences from the Protein Data Bank (Brookhaven) were analyzed using a computer program ("coiled-coil"; Lupas et al., (1991)). A score of 1.6 was obtained for residues 54-81 of HA2.

Inspection of the complete HA2 sequence revealed a continuous heptad repeat from residues 38 to 125. Although an earlier analysis had predicted a coiled coil structure for much of the 88-residue sequence of HA2 identified here, the register of the heptad repeat was not maintained throughout this sequence, and several interruptions in the coiled coil were also predicted (Ward and Dopheide, 1980; see also, Chambers et al., (1990)).

Peptide Synthesis and Purification

Peptides were synthesized using FMOC chemistry on an Applied Biosystems model 430A peptide synthesizer with Fastmoc reaction cycles modified to include acetic anhydride capping (reviewed in Kent, S.B.H., Ann. Rev. Biochem. 57:957-989 (1988); Fields et al., CITE (1991)). LOOP-36 corresponds to residues 54-89 and LOOP-52 corresponds to residues 38-89 of HA2 from the X-31 strain of influenza virus (Kilburne, CITE (1969)). The N-termini of LOOP-36 and LOOP-52 are acetylated and the C-termini are amidated. The peptides were cleaved using standard FMOC protocols and desalted on a Sephadex G-10 or G-25 column (Pharmacia) in 5% acetic acid. Final purification was by reverse-phase, high-

performance liquid chromatography (HPLC, Waters, Inc.) at 25°C using a Vydac preparative or semi-preparative C18 column (stock # 218TP1022 or 218TP510, respectively). A linear acetonitrile-H_2O gradient of 0.1% buffer B increase per minute was used with a flow rate of 10 ml/min (5ml/min for the semipreparative column). Buffer A is 0.1% TFA in water; buffer B is 90% acetonitrile and 0.1% TFA in water. The identity of the peptides was confirmed by laser desorption mass spectrometry (Finigan MAT LASERMAT). The measured mass for LOOP-36 was 4451 Da (expected 4450 Da), and for LOOP-52 was 6147 Da (expected 6146).

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy (for review, see Woody, CITE (1985)) was performed on an AVIV CD spectrophotometer (model 62DS) equipped with a thermoelectric controller. The cuvettes used for thermal unfolding studies and pH studies were 1 cm and 0.1 mm in pathlength and the cuvettes used for wavelength spectra were 1 mm and 0.1 mm in pathlength. Peptide concentration was measured by tyrosine absorbance at 275.5 nm (Edelhoch, H., Biochem. 6:1948-1954 (1967)), in 6 M guanidium chloride (Schwartz/Mann Biotech, Ultra-Pure grade).

The pH-dependence of Loop-36 structure was determined by monitoring the CD signal at 222nm. Measurements were made at 0°C and 32 μM peptide in a buffer of 20 mM (each) of sodium borate, sodium citrate and sodium phosphate, and 150 mM NaCl, adjusted to the correct pH with NaOH and HCl. Both transitions observed in the pH-dependence studies are >95% reversible (data not shown).

For thermal unfolding experiments, the CD signal was monitored at 222 nm as a function of temperature. Solutions at pH 7.0 contained 32 μM LOOP-36, 150 mM NaCl and 10 mM $NaPO_4$; or 500 μM Loop-52, 50 nM $NaPO_4$ and 150 nM NaCl. LOOP-52 pH 4.8 samples also contained 10 mm sodium citrate and 10 mm sodium borate. All thermal unfolding experiments are reversible (>95% for LOOP-36 samples and >87% for LOOP-52 samples) in temperature range from 0-60°C for LOOP-36, and

0-85% for LOOP-52.

CD spectra were obtained at 0°C in the same conditions as thermal unfolding experiments, except that the peptide concentration was 100 μM for LOOP-36. Percent helicity was calculated assuming that 100% helicity corresponds to -33,000 deg cm^2 $dmol^{-1}$ for LOOP-36, as has been found in studies of helical peptides of this length (Chen at al., CITE (1974)).

Equilibrium Sedimentation

The molecular weight was determined at 1°C for LOOP-36 and 4°C for LOOP-52, by analytical ultracentrifugation (reviewed in Lave et al., CITE (1992)), with a Beckmann XL-A Optima Analytical Ultracentrifuge equipped with absorbance optics. An An-60Ti rotor was used at 22,000 and 27,000 rpm. Experiments were performed over at least a 10-fold concentration range, at pH 7.2, and pH 4.87. LOOP-36 samples were made at three concentrations (300, 100, and 33 μM) per pH and LOOP-52 samples were made at six concentrations (500, 167, 56, 130, 43 and 14 μM) per pH, and all samples were exhaustively dialyzed (approximately 24 hours) against buffer prior to the experiments. Buffer conditions were 50 mM sodium phosphate, 150 mM NaCl, pH 7.2; or 5 mM sodium acetate, 150 mM NaCl, pH 4.7. The average molecular weight was determined by a simultaneous fit of the data using a non-linear least squares fit algorithm, HID-4000 (Johnson et al., CITE, (1981)).

B. Assessment of LOOP-36

LOOP-36 is a peptide which corresponds to the loop region of HA2 in the pH-neutral structure. It was synthesized and analyzed, using the methods described above, as to its conformation at the pH of membrane fusion (low pH, approximately 5.0). Results of these analyses are presented in Figures 5-8.

The structure of 100 μM LOOP-36 was monitored at 0°C by circular dichroism spectroscopy at pH 2, pH 4.8 and pH 7. The shape of the spectra reveals structure of approximately

50% helicity at pH 2, ≥80% helicity at pH 4.8 and random coil at pH 7. Units of helicity (molar ellipticity at 222 nm) are plotted on the y axis; for a peptide of this length, 100% helicity is -33,000 units of molar ellipticity at 222 nm. Results are represented graphically in Figure 5.

Thermal unfolding of LOOP-36 was assessed at pH 7 and pH 4.8. Cooperative unfolding of a highly helical structure is seen at pH 4.8, while no unfolding transition is seen at pH 7. (See Figure 6) Unfolding of 32 μM LOOP-36 is monitored by CD as a function of temperature. Units of helicity (molar ellipticity at 222 nm) are plotted on the y axis; for a peptide of this length, 100% helicity is -33,000 units of molar ellipticity at 222 nm.

The pH-dependence of the LOOP-36 folded structure was monitored by measuring molar ellipticity at 222 nm as a function of pH. (See Figure 7) At 0°C, 32 μM LOOP-36 shows two folding transitions with change in pH; from pH 7 (and higher) to pH 5 and from pH 4 to pH 2. The peptide is unfolded at pH 7 and above, ≥80% helical between pH 4 and pH 5 and 50% helical at pH 2.

Molecular weight determinations of LOOP-36 were carried out. Results are shown in Figure 8. Representative equilibrium sedimentation data show linearity of ln A_{232} versus r^2, indicating a well-behaved, single species at both pH 7.2 and pH 4.7. The slopes are approximately 3-fold different, as expected if the pH 7.2 species is monomeric and the pH 4.7 species is trimeric (see Table).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

The invention claimed is:

1. A method of reducing infection of host cells by an enveloped virus, comprising contacting the enveloped virus with an agent that prevents a conformational change of the fusion protein of the enveloped virus, the fusion protein having a coiled-coil conformation and the conformational change resulting in extension of the fusion protein toward the host cell membrane.

2. A method of reducing infection of mammalian host cells by influenza virus, comprising contacting the influenza virus with an agent that alters the conformational change undergone by influenza virus hemagglutinin, the conformational change comprising extension of the fusion protein away from the viral envelope and toward the membrane of an endosome.

3. The method of Claim 2, wherein the influenza virus is in the mammalian host cells in the late endosome.

4. The method of Claim 3, wherein the agent alters the conformational change by maintaining the pH of the late endosome at a higher pH than that normally present in the late endosome.

5. The method of Claim 3, wherein the agent alters the conformational change by stabilizing the metastable conformation of influenza virus hemagglutinin, thus preventing membrane fusion.

6. The method of Claim 2, wherein the agent alters the conformational change by causing the conformational change to occur sooner after entry into the mammalian

cells than it would occur in the absence of the agent and resulting in an irreversible conformational change in hemagglutinin.

7. A method of reducing infection of mammalian host cells by the HIV virus, comprising contacting the HIV virus with an agent that alters the conformational change undergone by the HIV virus fusion protein, the conformational change comprising extension of the fusion protein away from the viral envelope and toward the membrane of the host cell.

8. The method of Claim 7, wherein the agent alters the conformational change by stabilizing the metastable conformation of the HIV virus fusion protein, thus preventing membrane fusion.

9. A method of enhancing membrane fusion between two membranes, comprising contacting a fusion protein present on the first of the two membranes with an agent that enhances conformational change of the fusion protein, the conformational change resulting in extension of the fusion protein away from the first membrane and toward the surface of the second membrane.

10. The method of Claim 9, wherein the membranes to be fused are cellular membranes.

11. The method of Claim 9, wherein the first membrane is the cellular membrane of a sperm cell, the second membrane is the cellular membrane of an ovum, and enhanced membrane fusion results in fertilization of the ovum.

12. A method of identifying an agent which reduces the conformational change of the fusion protein of a virus, comprising the steps of:

a) combining the agent and the fusion protein in conditions under which the fusion protein is in the metastable state;

b) subjecting the combination produced in (a) to conditions under which, in the absence of the agent, the fusion protein changes conformation to the fusogenic state; and

c) determining whether the protein undergoes a conformational change from the metastable state to the fusogenic state,

whereby if the fusion protein does not undergo a conformational change in the presence of the agent or undergoes a different conformational change in the presence of the agent than it normally undergoes in the absence of the agent, it is indicative of the ability of the agent to reduce the conformational change of the fusion protein.

13. A method of identifying an agent which reduces the conformational change of influenza virus in mammalian cells, comprising the steps of:

a) combining the agent and LOOP-36 peptide under conditions of neutral pH;

b) reducing the pH of the combination produced in (a) to pH approximately 5.0; and

c) determining whether LOOP-36 undergoes a conformational change from the metastable state to the fusogenic state,

whereby if LOOP-36 does not undergo a conformational change in the presence of the agent or undergoes a different conformational change in the presence of the agent than it normally undergoes at pH of approximately 5.0, it is indicative of the ability of the agent to reduce the conformational change of influenza virus.

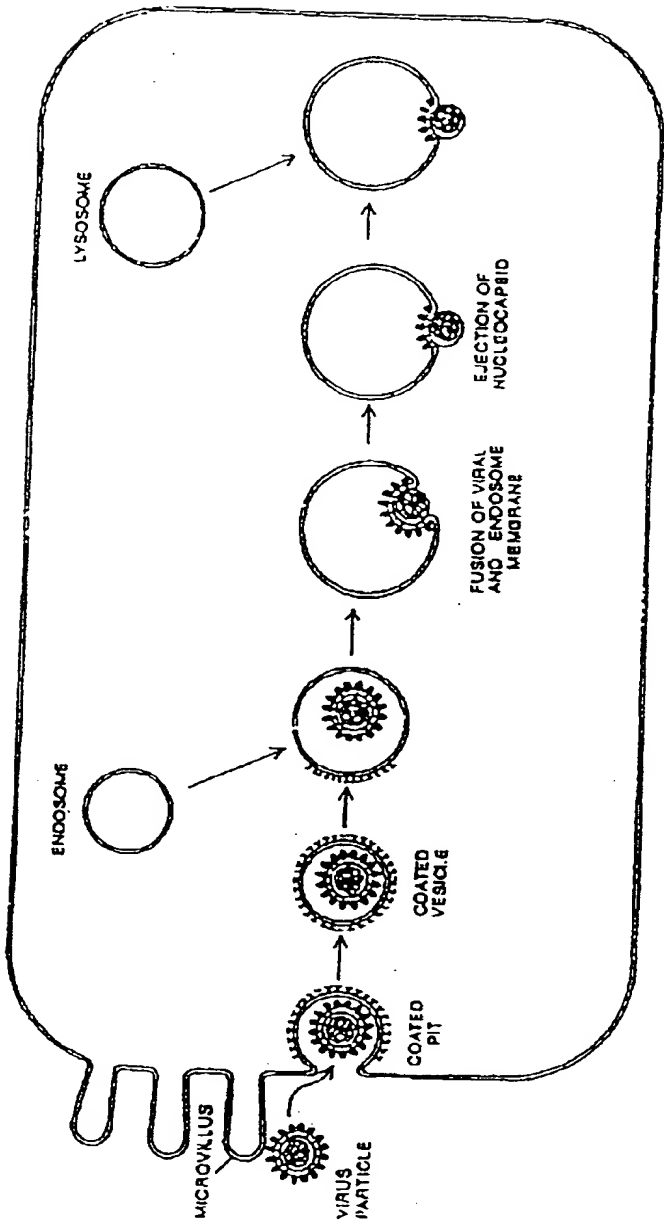
14. A method of identifying an agent which reduces the conformational change of influenza virus in mammalian cells, comprising the steps of:

a) combining the agent and LOOP-52 peptide under conditions of neutral pH and ambient temperature;

b) raising the temperature of the combination produced in (a) to approximately 60°C; and

c) determining whether LOOP-52 undergoes a conformational change from the metastable state to the fusogenic state,

whereby if LOOP-52 does not undergo a conformational change in the presence of the agent or undergoes a different conformational change in the presence of the agent than it normally undergoes at a temperature of approximately 60°C, it is indicative of the ability of the agent to reduce the conformational change of influenza virus.

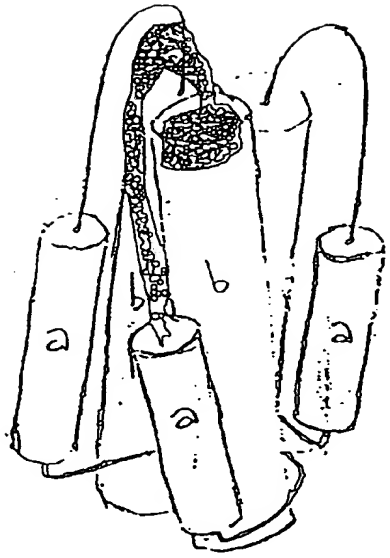


From: Fields, Virology (1990)

FIGURE 1

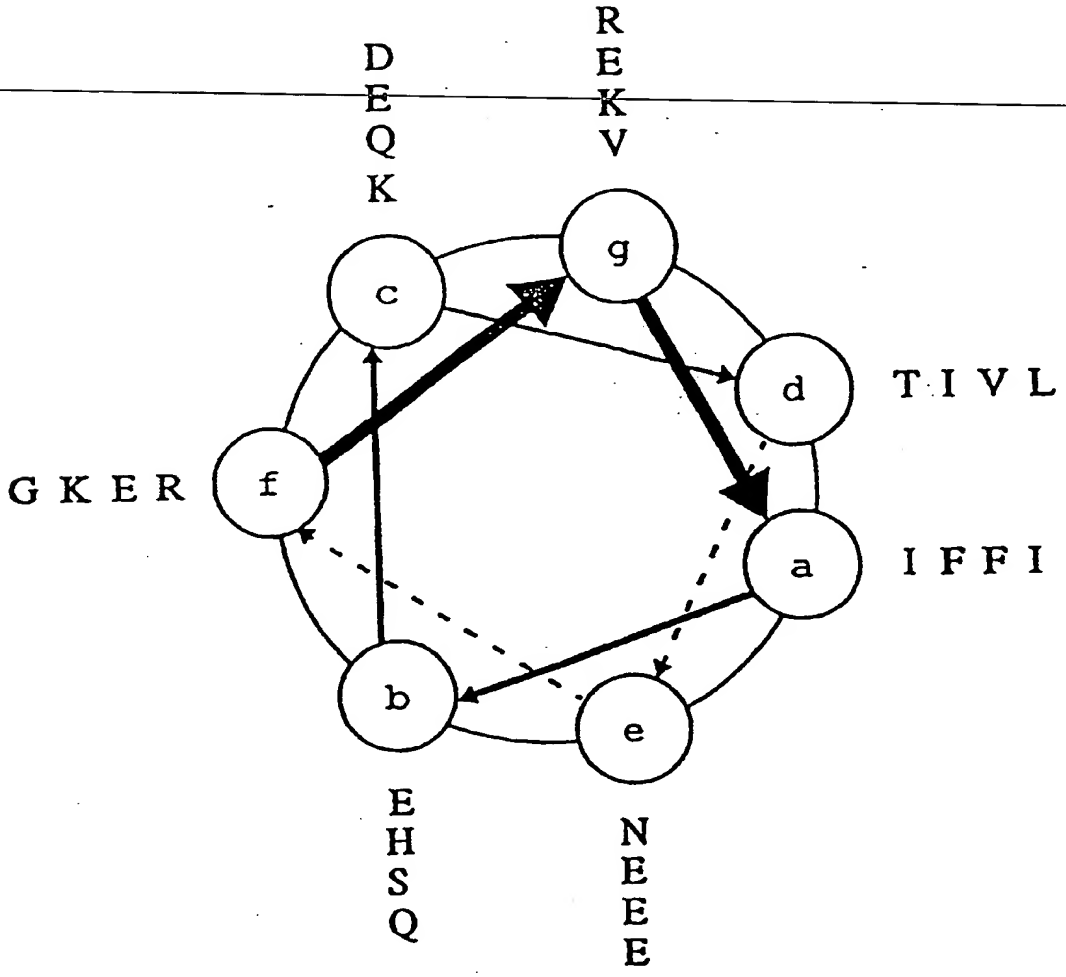
--Fusion Peptide--
bcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
NH2- GLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLNRV
--LOOP-36--
abcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
IEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLT
abcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
DSEMNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEAL
abcdefga
NNRFQIKG-COOH

FIGURE 2

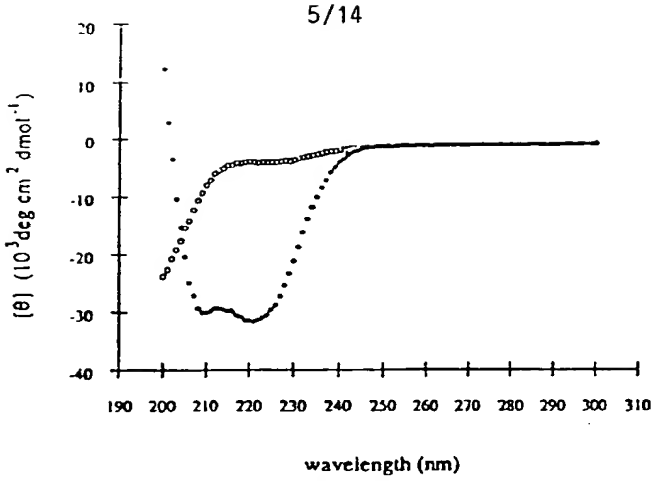


DORMANT STATE
HA_2

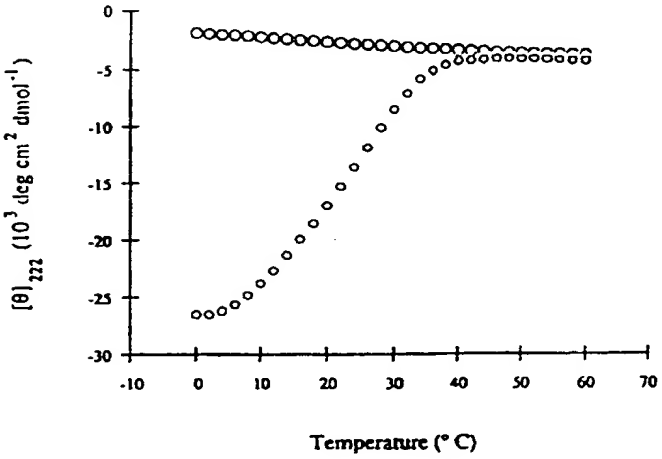
FIGURE 3


R
E
K
V
D
E
Q
K
g
c
d
T I V L
G K E R
f
a
I F F I
b
e
E
H
S
Q
N
E
E
E

A


[θ] (10^3deg cm^2 dmol^{-1})
20
10
0
-10
-20
-30
-40
190 200 210 220 230 240 250 260 270 280 290 300 310
wavelength (nm)

B


$[\theta]_{222}$ (10^3 deg cm^2 dmol^{-1})
0
-5
-10
-15
-20
-25
-30
-10 0 10 20 30 40 50 60 70
Temperature (° C)

C

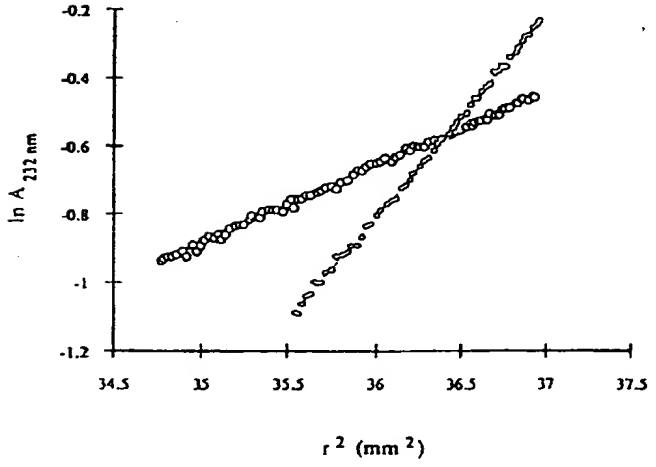

ln $A_{232\ nm}$
-0.2
-0.4
-0.6
-0.8
-1
-1.2
34.5 35 35.5 36 36.5 37 37.5
r^2 (mm^2)

FIGURE 6A

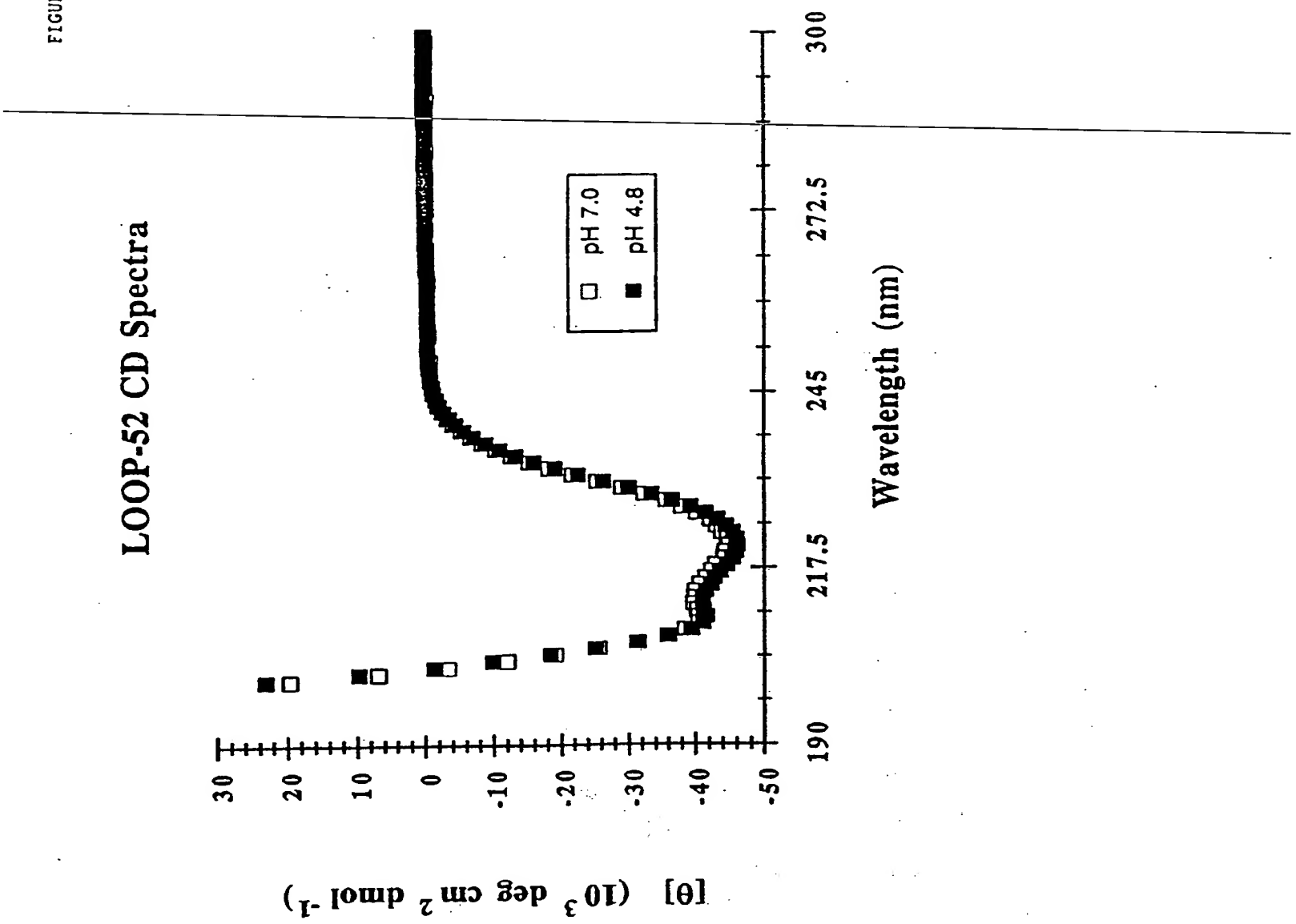

LOOP-52 CD Spectra
pH 7.0
pH 4.8
[θ] (10³ deg cm² dmol⁻¹)
30
20
10
0
-10
-20
-30
-40
-50
190
217.5
245
272.5
300
Wavelength (nm)

FIGURE 6B

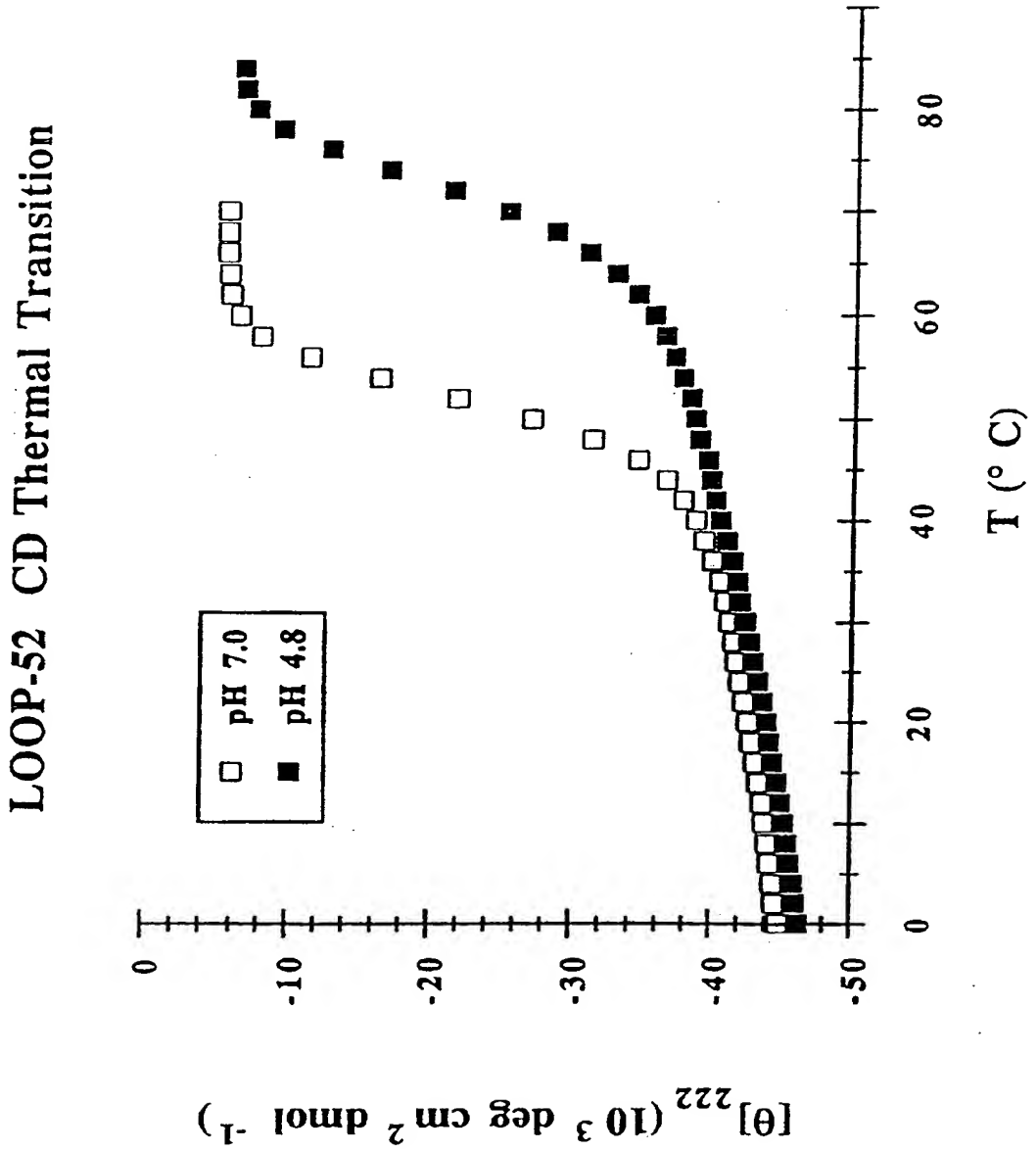
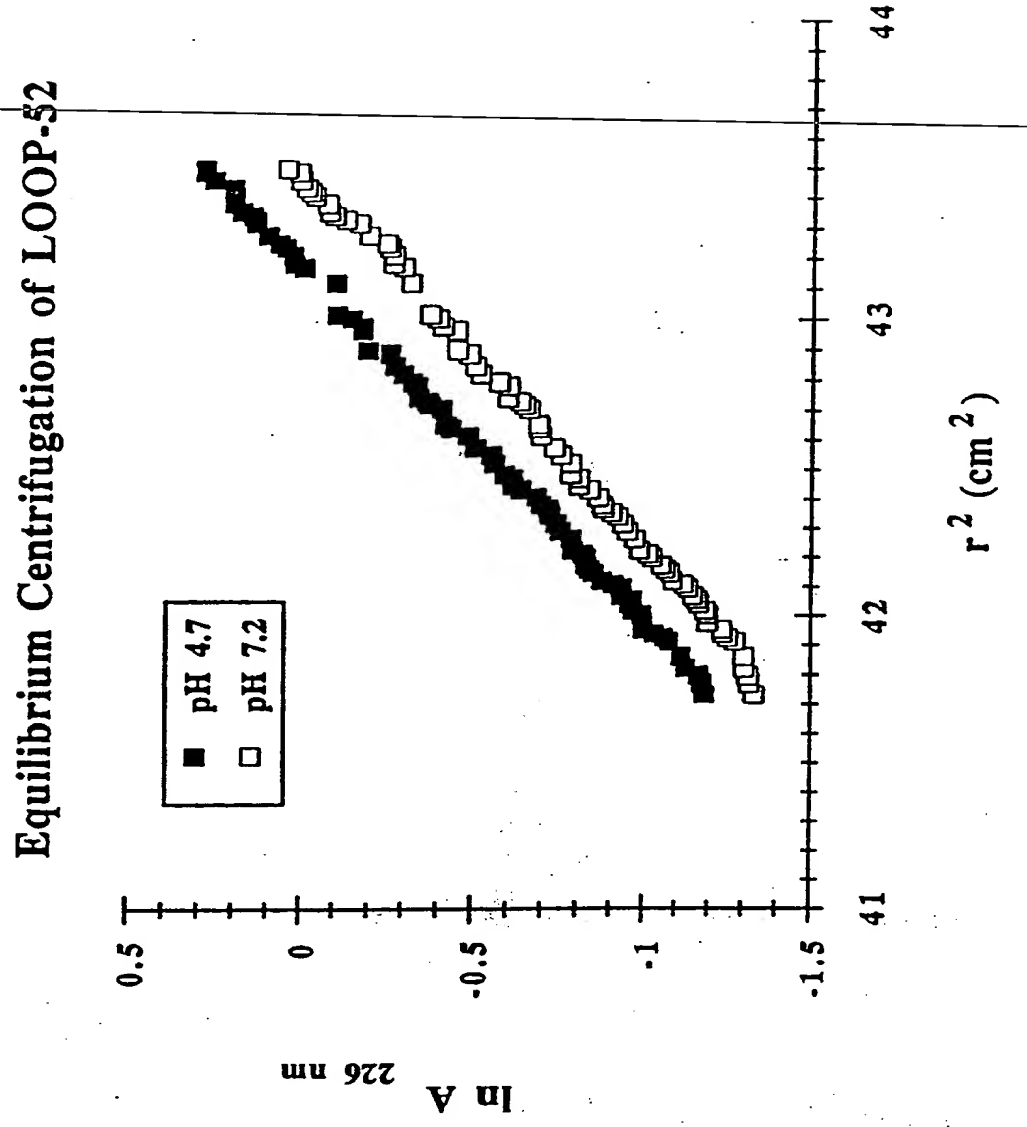

LOOP-52 CD Thermal Transition
pH 7.0
pH 4.8
0
-10
-20
-30
-40
-50
0
20
40
60
80
T (° C)
[θ]222 (10 3 deg cm 2 dmol -1)

FIGURE 6C


Equilibrium Centrifugation of LOOP-52
pH 4.7
pH 7.2
ln A 226 nm
0.5
0
-0.5
-1
-1.5
41
42
43
44
r 2 (cm 2)

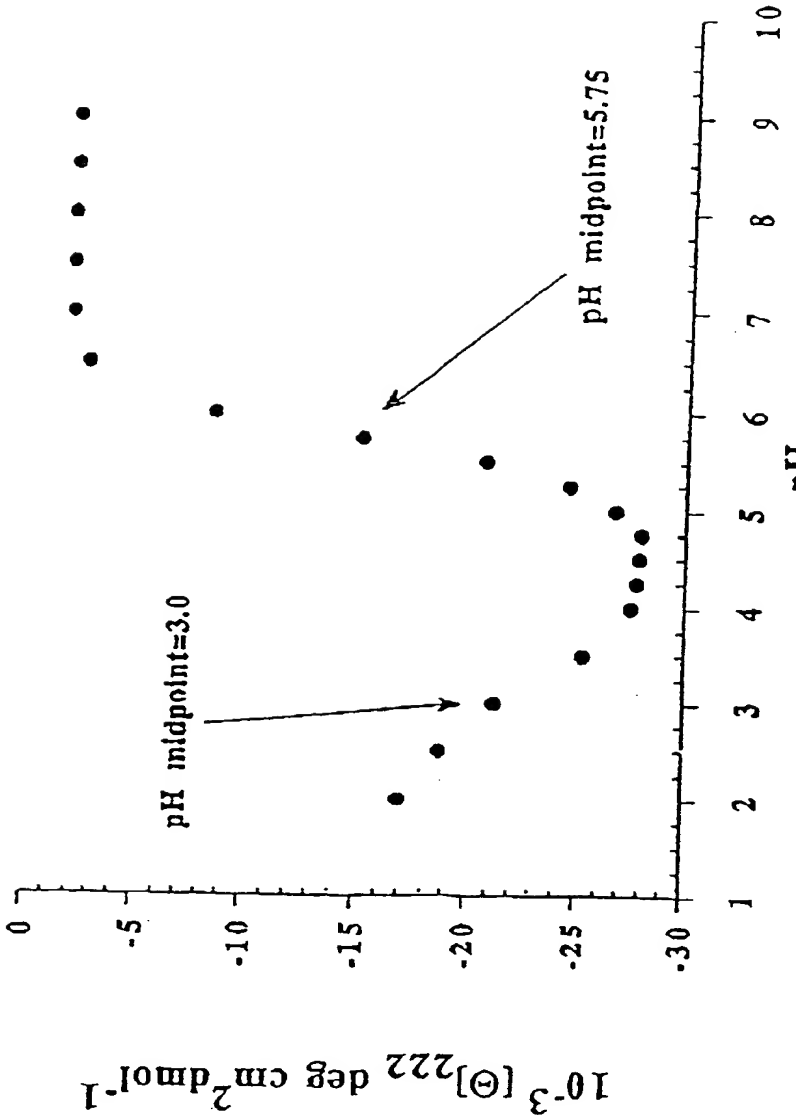

10^{-3} $[\Theta]_{222}$ deg $cm^2 dmol^{-1}$
0
-5
-10
-15
-20
-25
-30
1
2
3
4
5
6
7
8
9
10
pH
pH midpoint=3.0
pH midpoint=5.75

FIGURE 7

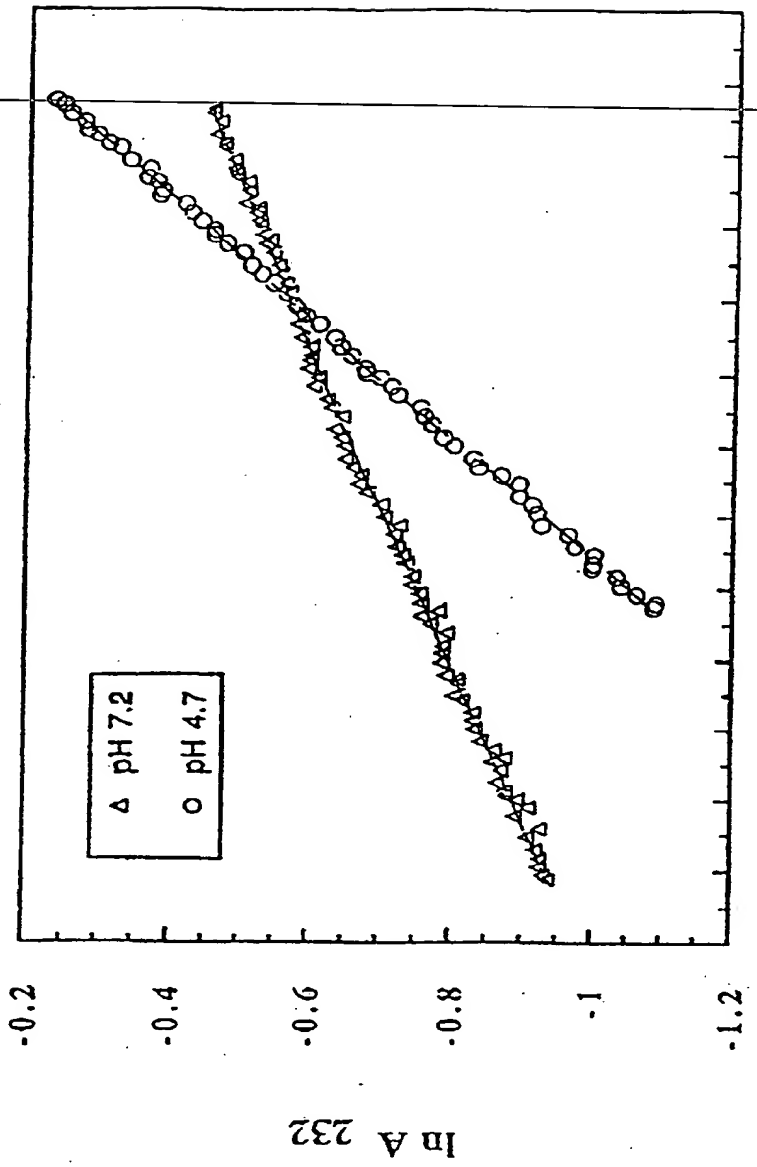


FIGURE 8

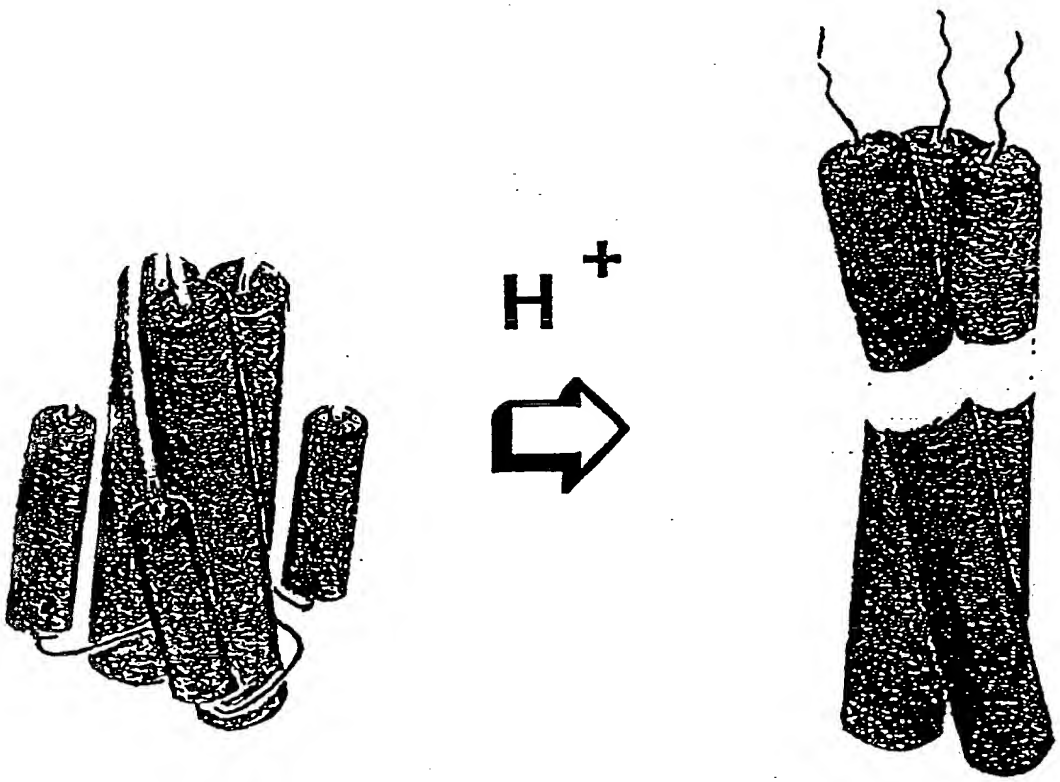
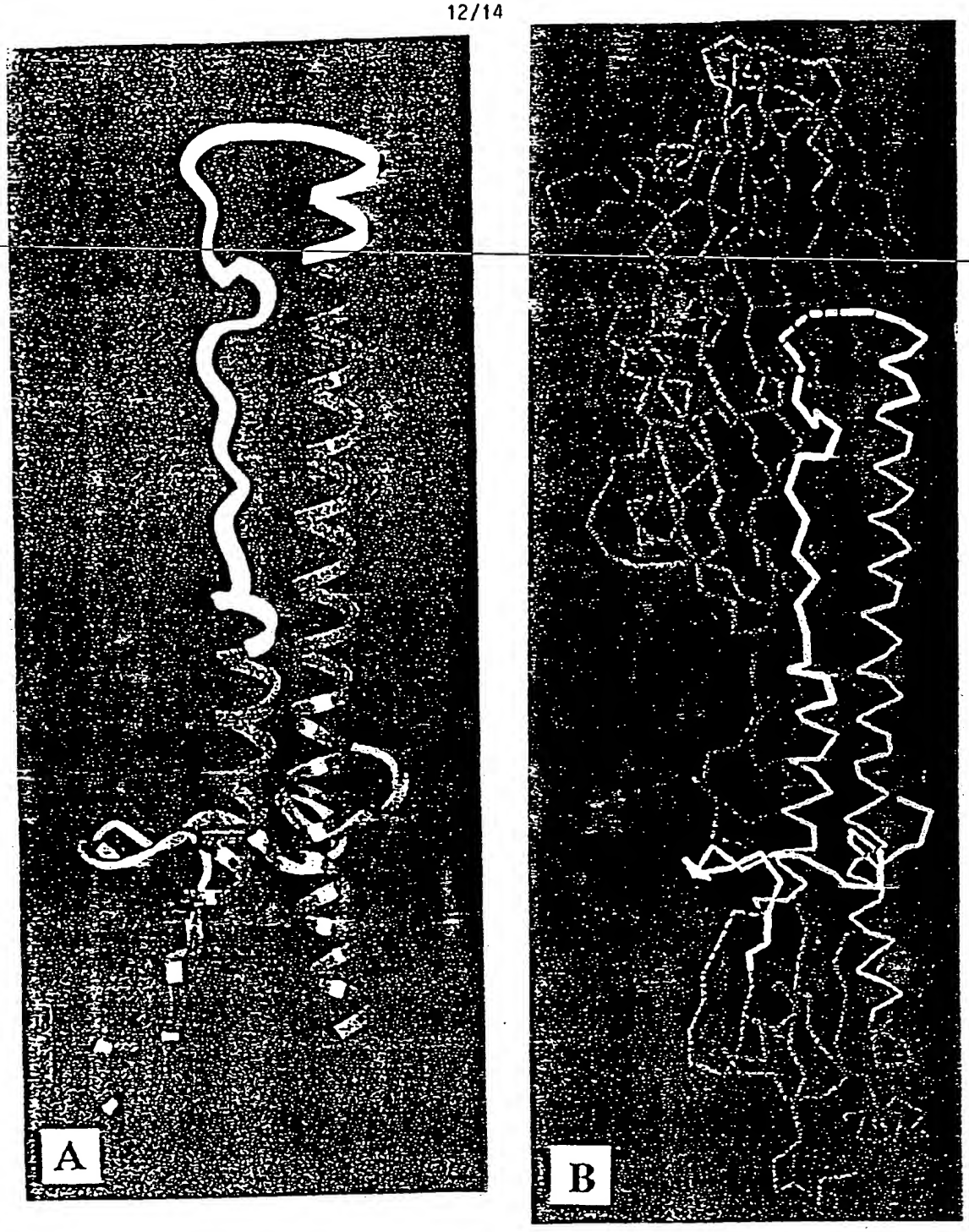

H +

FIGURE 10



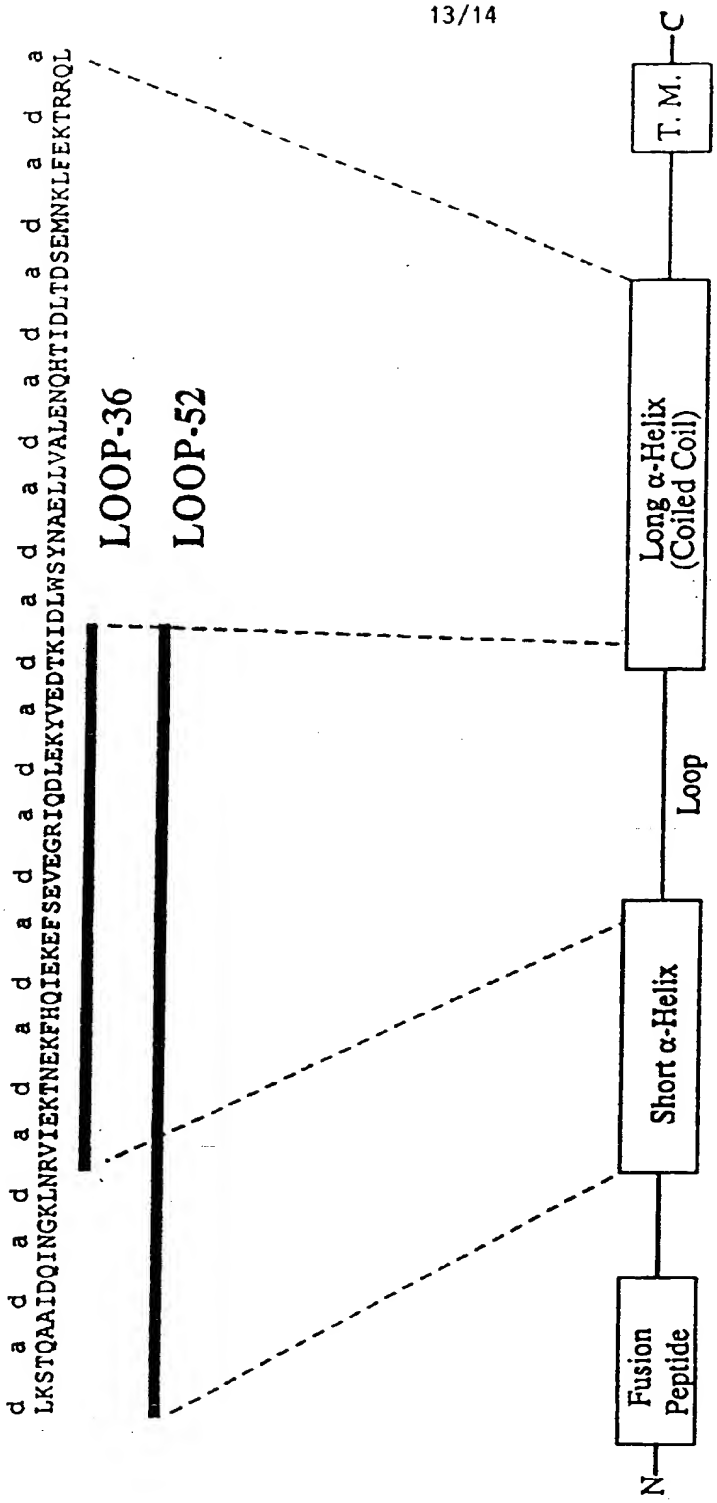
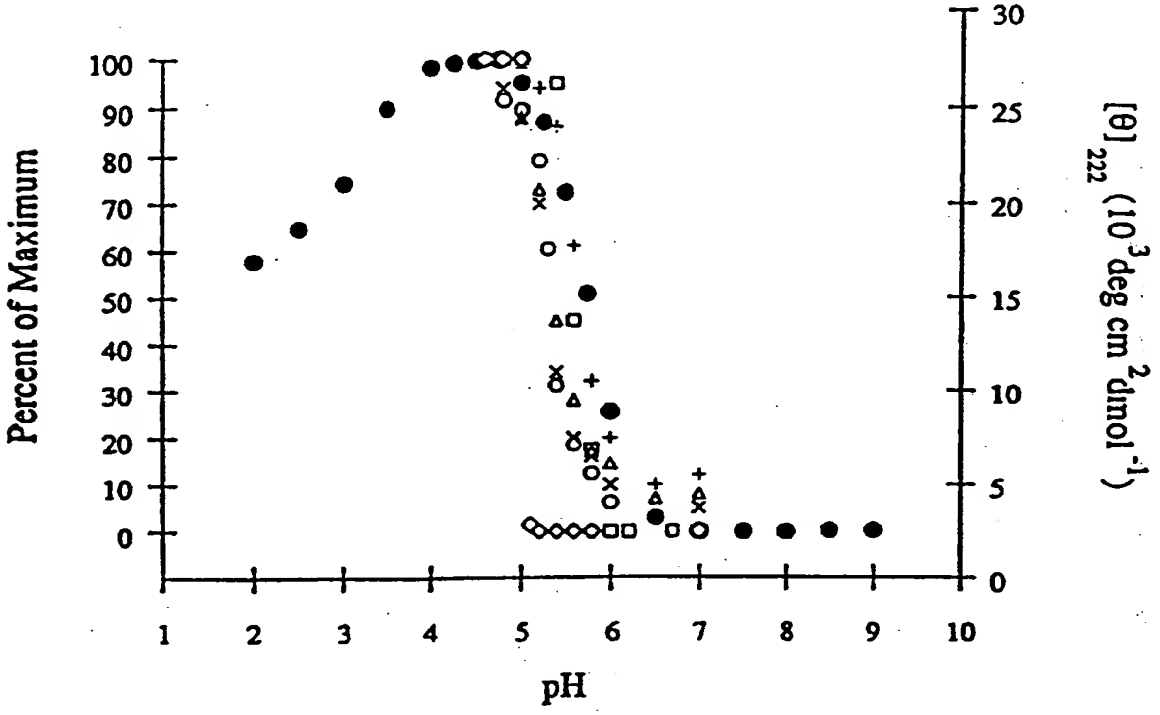

d a d a d a d a d a d a d a d a d a d a d a d a d a
LKSTQAAIDQINGKLNRVIEKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTRRQL
LOOP-36
LOOP-52
T. M.
C
Long α-Helix (Coiled Coil)
Loop
Short α-Helix
Fusion Peptide
N

FIGURE 11


Percent of Maximum
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
pH
30
25
20
15
10
5
0
$[\theta]_{222}$ (10^3deg cm ^{2}dmol $^{-1}$)

INTERNATIONAL SEARCH REPORT

	International application No. PCT/US94/00709

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL : 435/5, 6, 235.1, 236, 238; 530/300, 350; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : C12Q 1/70, 1/68; C12N 7/00, 7/06, C07K 5/00, 13/00; A61K 31/70
435/5, 6, 235.1, 236, 238; 530/300, 350; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, APS, PIR, SWIS-PROT, GEN-BANK

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 260, NO. 5, ISSUED 10 MARCH 1985, DOMS ET AL," MEMBRANE FUSION ACTIVITY OF INFLUENZA VIRUS HEMAGGLUTININ- THE LOW PH-INDUCED COMFORMATIONAL CLEAVAGE," PAGES 2973-2981, SEE ENTIRE DOCUMENT.	1-8 AND 12-14
Y	THE JOURNAL OF BIOLOGICAL CHEMISTY, VOLUME 263, NO. 9, ISSUED 25 MARCH 198, WHARTON ET AL, "CONFORMATIONAL ASPECTS OF THE ACID-INDUCED FUSION MECHANISM OF INFLUENZA VIRUS MEMAGGLUTININ-CIRCULAR DICHROISUM AND FLUORESCENCE STUDIES," PAGES 4474-4480, SEE ENTIRE DOCUMENT.	1-8 AND 12-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 MARCH 1994	Date of mailing of the international search report 25 APR 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ABDEL A. MOHAMED Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00709

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 264, NO. 7, ISSUED 05 MARCH 199, MORRIS ET AL, "KIRETICS OF PH DEPENDENT FUSION BETWEEN 3T3 FIBROBLASTS EXPRESSING INFLUENZA HEMAGGLUTININ AND RED BLOOD CELLS-MEASUREMENT BY DEQUENCHING OF FLUORESCENCE", PAGES 3972-3978, SEE ENTIRE DOCUMENT.	1-8 AND 12-14
Y	J.GEN. VIROL., VOLUME 69, ISSUED 1988, RUIGROK ET AL, "STUDIES OF THE STRUCTURE OF THE INFLEUENZA VIRUS HAEMAGGLUTININ AT THE PH OF MEMBRANE FUSION" PAGES 2785-2795, SEE ENTIRE DOCUMENT.	1-8 AND 12-14
Y	J. CELL BIOL., VOLUME 105, ISSUED 1987, WHITE ET AL, "ANTI-PEPTIDE ANTIBODIES DETECT STEPS IN A PROTEIN CONFORMATIONAL A CHANGE: LOW-PH ACTIVATION OF THE INFLUENZA VIRUS HEMOGGLUTININ," PAGES 287-2896, SEE ENTIRE DOCUMENT.	9-11
Y	J. CELL BIOL., VOLUME 104, ISSUED 1987, PRIMAKOFF ET AL.," INDENTIFICATION AND PURIFICATION OF A SPERM SURFACE PROTEIN WITH A POTENTIAL ROLE IN SPERM-EGG MEMBRANE FUSION", PAGES 141-149, SEE ENTIRE DOCUMENT.	9-11
Y	NATURE, VOLUME 356, ISSUED 19 MARCH 1992, BLOBEL ET AL, "A POTENTIAL FUSION PEPTIDE AND AN INTEGRIN LIGAND DOMAIN IN A PROTEIN ACTIVE IN SPERM-EGG FUSION", PAGES 248-252, SEE ENTIRE DOCUMENT.	9-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00709

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/70, 1/68; C12N 7/00, 7/04, 7/06, C07K 5/00, 13/00; A61K 31/70, 435/5, 6, 235.1, 236, 238; 530/300, 350; 514/44